

Differences in Incubation Length and Hatchling Morphology among Five Species of Oviparous *Phrynocephalus* Lizards (Agamidae) from China

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Abstract We incubated eggs of five *Phrynocephalus* species (*P. albolineatus*, *P. axillaries*, *P. grumgrzimaloi*, *P. helioscopus* and *P. przewalskii*) at three constant temperatures (24 °C, 28 °C and 32 °C) to examine differences in incubation length and hatchling morphology among species and among temperature treatments. We combined data from this study with those reported previously for *P. frontalis* and *P. versicolor* to examine whether embryonic stage at laying is a causal factor for interspecific variation in incubation length, and whether the phylogenetic relationship inferred from hatchling morphology is consistent with the relationship based on mitochondrial DNA data. Mean values for incubation length differed among the five species studied herein and, in all these five species, incubation length decreased at a decreasing rate as temperature increased. In none of the five species did hatchling size (snout-vent length and body mass) and other morphological variables differ among the three temperature treatments. The seven oviparous *Phrynocephalus* lizards found in China differ from each other in hatchling morphology, and embryonic stage at laying is a causal factor of inter- and intra-specific variation in incubation length. The phylogenetic relationship inferred from hatchling morphology is not always consistent with the currently known relationship based on mitochondrial DNA data. Data from this study and those reported previously allow the conclusion that any *Phrynocephalus* species may have its unique position along the axis defined by hatchling morphology.

Keywords Agamidae, *Phrynocephalus* lizards, oviparous species, egg incubation, incubation length, hatchling morphology

1. Introduction

Toad-headed lizards of the reproductively bimodal genus *Phrynocephalus* (Agamidae) inhabit desert, arid or semiarid habitats in Central and West Asia and North-Northwest China, westwards to southern Jordan and eastwards to Hebei province of northern China, with all viviparous species restricted to the Qinghai-Tibet Plateau of China and adjacent regions (Zhao, 1999; Barabanov and Ananjeva, 2007; Ji *et al.*, 2009; Noble *et al.*, 2010). Environmental temperatures across the region occupied

by toad-headed lizards are characterized by a low mean and great amplitude of thermal fluctuations (Wang *et al.*, 2013a). This group is among the animal taxa whose taxonomy is very confusing. Recent studies have shed considerable light on the systematics of this group, but also resulted in considerable changes in nomenclature. Far from there being 140 species, as was widely believed until the 1990s, only some 40 *Phrynocephalus* species are currently recognized to be valid (Barabanov and Ananjeva, 2007). China is currently known to have six viviparous and six (if not including *P. frontalis*) or seven oviparous species of toad-headed lizards if not including invalid synonyms, with the potential of more species to be discovered or redescribed (Wang and Fu, 2004; Barabanov and Ananjeva, 2007; Gozdzik and Fu, 2009).

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Received: 9 October 2013 Accepted: 15 December 2013

To solve systematic and nomenclatural issues of this group, recent studies use not only traditional (morphology, morphometrics and life-history: Wang *et al.*, 1996, 1999; Qu *et al.*, 2011a; Wang *et al.*, 2011; Sun *et al.*, 2012; Jin *et al.*, 2013; Zeng *et al.*, 2013) but also modern (cytogenetics, protein electrophoresis, molecular phylogenetics and ethology: Wang *et al.*, 2002; Wang and Fu, 2004; Guo and Wang, 2007; Noble *et al.*, 2010; Li *et al.*, 2011; Qi *et al.*, 2011) methodologies. Other studies conducted over the past few years have provided information on egg incubation or embryonic development, offspring phenotype and thermal physiology (Shu *et al.*, 2010; Qu *et al.*, 2011b, c; Tang *et al.*, 2012; Wang *et al.*, 2013b; Zeng *et al.*, 2013), which is also useful to the workers on this genus. Recent studies overall support the idea that any *Phrynocephalus* species or population may have its unique position along the morphological, morphometric, behavioral, molecular, physiological and/or life-history axes.

Here, we describe a study incubating eggs of five oviparous toad-headed lizards (*P. albolineatus*, *P. axillaries*, *P. grumgrzimailoi*, *P. helioscopus* and *P. przewalskii*) at three constant temperatures (24 °C, 28 °C and 32 °C) to examine differences in incubation length and hatchling morphology among species and among temperature treatments. Eggs of the former four species have never been incubated at any known temperature, whereas eggs of *P. przewalskii* have been incubated at multiple temperatures ranging from 26–34 °C (Tang *et al.*, 2012; Zeng *et al.*, 2013). *Phrynocephalus frontalis* and *P. versicolor* are another two oviparous species whose eggs have been incubated at multiple temperatures ranging from 24–34 °C (Qu *et al.*, 2011b; Tang *et al.*, 2012). Though differing morphologically at hatching (see below) and during adulthood (Zhao *et al.*, 1999), *P. albolineatus* and *P. grumgrzimailoi* have been proposed to be invalid synonyms of *P. guttatus* (Barabanov and Ananjeva, 2007). Furthermore, *P. frontalis* has been proposed to be an invalid synonym of *P. przewalskii* (Wang and Fu, 2004; Barabanov and Ananjeva, 2007; Gozdzik and Fu, 2009). Data from this study and those reported previously allow us to address the following four questions: (1) Does incubation length vary among species? (2) If so, is embryonic stage at laying a causal factor for such variation? (3) Do the species involved differ from each other in hatchling morphology? (4) If so, is their phylogenetic relationship inferred from morphological data consistent with the currently known relationship inferred from mitochondrial DNA data?

2. Materials and Methods

2.1 Animal collection and maintenance Lizards in this study were collected in May of 2010–2011 from Tacheng (*P. albolineatus*; 46°44' N, 82°58' E), Yanqi (*P. axillaries*; 42°03' N, 86°34' E), Qitai (*P. grumgrzimailoi*; 44°01' N, 89°35' E) and Yumin (*P. helioscopus*; 46°12' N, 82°59' E) in Xinjiang, and Wuwei (*P. przewalskii*; 37°56' N, 102°38' E) in Gansu. Females with yolking follicles or oviductal eggs were transported to our laboratory in Hangzhou, where between 7–10 females of the same species were housed in each 900 mm × 650 mm × 600 mm (length × width × height) communal cage with a substrate of sand (150 mm depth) and pieces of clay tiles as shelter. All communal cages were placed in a room where temperatures varied from 20–28 °C. Thermoregulatory opportunities were provided between 07:00–19:00 h using a 100 w full-spectrum lamp (EuroZoo, Germany); overnight temperatures followed room temperatures. Mealworms (larvae of *Tenebrio molitor*) and house crickets (*Achetus domesticus*) dusted with multivitamins and minerals and water were provided daily, so that excess food and water were always available to females in the communal cages.

Females with shelled oviductal eggs were removed from the communal cages, and individually housed in 200 mm × 200 mm × 200 mm egg-laying cages with a substrate of moist sand (50 mm depth) and a 25 w spotlight mounted in each cage to allow behavioral thermoregulation. Post-oviposition females were measured (to the nearest 0.1 mm) for snout-vent length (SVL) and tail length and weighed (to the nearest 10 mg) before they were returned to the communal cages, where they remained until release to the field in late August.

2.2 Egg collection and incubation Females of the five species all laid pliable-shelled eggs between late May and early July. Eggs were collected, measured (to the nearest 0.01 mm) for length and width with a digital caliper (Mitutoyo, Japan), and weighed (to the nearest 1 mg) on a Mettler balance always less than 3 h post-laying, thereby minimizing the uncertainty about the egg mass at laying due to loss or gain of water.

A total of 251 (28 *P. albolineatus*, 31 *P. axillaries*, 64 *P. grumgrzimailoi*, 25 *P. helioscopus* and 103 *P. przewalskii*) eggs were either dissected for identification for Dufaure and Hubert's (1961) embryonic stage (hereafter DH stage) at laying or incubated at one of three constant temperatures (24 °C, 28 °C and 32 °C). The incubated eggs were individually placed in covered plastic jars (50 ml) with known amounts of vermiculite and water at

about -220 kPa water potential (1 g dried vermiculite: 1 g water; Ji and Braña, 1999). Each incubated egg was half-buried lengthwise in the substrate, with the surface near the embryo exposed to air inside the jar. Jars were randomly assigned to three Binder KB incubators (Binder, Germany), with temperatures set at 24°C , 28°C and 32°C , respectively. We rotated jars every other day following a predetermined schedule to minimize any possible effects of thermal gradients inside the incubator. Water potential was adjusted at the same time interval by weighing jars, and water was added into the substrate to compensate for evaporative losses and water taken up by eggs. Incubation length, the time interval from laying to pipping, was recorded for each hatched egg.

2.3 Size and morphology of hatchlings Hatchlings were collected, weighed (to the nearest 1 mg) and measured (to the nearest 0.01 mm) with a digital caliper. Measurements taken for each hatchling included: SVL, tail length, abdomen length (from the posterior base of the forelimb to the anterior base of the hindlimb), head length (from the snout to the posterior end of the skull), head width (taken at the posterior end of the mandible), forelimb length (humerus plus ulna) and hindlimb length (femur plus tibia) (Zhang *et al.*, 2005).

2.4 Statistical analyses We used Statistica version 6.0 for PC (StatSoft Inc., USA) to analyze data. We pooled data for both sexes because preliminary analyses revealed that none of the examined variables differed between the sexes within each species. Prior to parametric analyses, data were tested for normality using the Kolmogorov-Smirnov test and, for variance homogeneity, using the Bartlett's test. We used linear regression analysis to examine whether an examined variable was related to egg mass (for hatchling SVL and mass) or hatchling SVL (for other hatchling morphological variables). We used two-way ANOVA to examine whether egg mass and incubation length differed among species and among temperature treatments. We used one-way ANCOVA with egg mass or hatchling SVL as the covariate to examine whether an examined hatchling variable differed among species and among temperature treatments. A principal components analysis (PCA) based on seven hatchling morphological variables was performed to show positions of the seven oviparous *Phrynocephalus* lizards (including *P. frontalis* and *P. versicolor* studied previously; Qu *et al.*, 2011b) found in China on a two-dimensional plane; size effects on the traits other than SVL were removed using residuals from the regressions of corresponding variables on SVL. We used one-way ANOVA to examine whether

PCA scores on the first two axes differed among species. PCA scores on the first two axes also were used to establish a tree showing the phylogenetic relationship of the seven species based on morphological data. Tukey's test was performed on the traits that differed among species or among temperature treatments. Throughout this paper, descriptive statistics are presented as mean \pm standard error (SE) and range, and the significance level is set at $\alpha = 0.05$.

3. Results

Eggs weighed between 526–758 mg in *P. albolineatus*, 487–711 mg in *P. axillaries*, 450–938 mg in *P. grumgrzmailoi*, 612–858 mg in *P. helioscopus*, and 531–1016 mg in *P. przewalskii*. Mean values for egg mass differed among the five species ($F_{4, 142} = 7.09$, $P < 0.0001$), but not among the three temperature treatments ($F_{2, 142} = 1.27$, $P = 0.283$); the species \times temperature interaction was not a significant source of variation in egg mass ($F_{8, 142} = 1.89$, $P = 0.067$). Statistically, the mean egg mass did not differ between *P. helioscopus* and *P. przewalskii* (Tukey's test; $P = 0.997$), nor among the other three species (Tukey's test; all $P > 0.337$). Mean values for egg mass were significantly greater in *P. helioscopus* (705 mg) and *P. przewalskii* (718 mg) than in *P. albolineatus* (626 mg), *P. axillaries* (590 mg) and *P. grumgrzmailoi* (649 mg) (Tukey's test; all $P < 0.021$).

Embryonic stages at laying overall varied between DH stage 28–32 (Table 1). Mean values for incubation length differed among the five species ($F_{4, 142} = 15.39$, $P < 0.0001$) and among the three temperature treatments ($F_{2, 142} = 934.28$, $P < 0.0001$); the species \times temperature interaction was a significant source of variation in incubation length ($F_{8, 142} = 5.60$, $P < 0.0001$). Statistically, the mean incubation length did not differ between *P. axillaries* and *P. grumgrzmailoi* (Tukey's test; $P = 0.984$), nor among the other three species (Tukey's test; all $P > 0.381$). Mean values for incubation length overall were longer in *P. axillaries* and *P. grumgrzmailoi* than in the other three species (Tukey's test; all $P < 0.041$). Incubation length was nonlinearly sensitive to incubation temperature in all five species. At the changeover from 24°C to 28°C the mean incubation length was shortened by about 11 d in *P. albolineatus*, *P. axillaries* and *P. grumgrzmailoi*, 12 d in *P. przewalskii*, and 15 d in *P. helioscopus*; at the changeover from 28°C to 32°C , the mean incubation length was shortened by about 7 d in *P. albolineatus* and *P. przewalskii*, 8 d in *P. grumgrzmailoi* and *P. helioscopus*, and 10 d in *P. axillaries* (Table 1).

In none of the five species did hatchling size (SVL and mass) and other examined variables differ among the three temperature treatments after accounting for egg mass (for hatchling size and mass) or hatchling SVL (for other variables) (ANCOVA; all $P > 0.091$). Data pooled for the three treatments showed that the five species differed from each other in hatchling morphology (Figure 1, Table 2).

The PCA on data from the seven oviparous *Phrynocephalus* lizards resolved two components (with eigenvalues ≥ 1) from seven hatchling variables, accounting for 82.4 % of the variation in the original data (Table 3). The first component (71.7 % variance explained) had high positive loadings for all seven variables examined, and the second explained 10.7 % of the total variance (Table 3). Factor scores on the first two axes differed among the seven species (Table 3) and, as expected, each species had a unique position on a two-dimensional plane (Figure 2). Morphologically, the differences between *P. helioscopus* and the other six species were greater than those between any other species pair, and [*P. albolineatus* and *P. przewalskii*], [*P. axillaries* and *P. grumgrzimailoi*], and [*P. frontalis* and *P. versicolor*] were the three reciprocally most related species pairs (Figure 3).

4. Discussion

Data from this study and those reported previously for *P. frontalis*, *P. przewalskii* and *P. versicolor* consistently show that incubation length decreases at an ever-decreasing rate as temperature increases across the range where successful embryonic development can take place (Table 1; Qu *et al.*, 2011b; Tang *et al.*, 2012). Such a relationship between incubation length and temperature is widespread among oviparous reptiles,

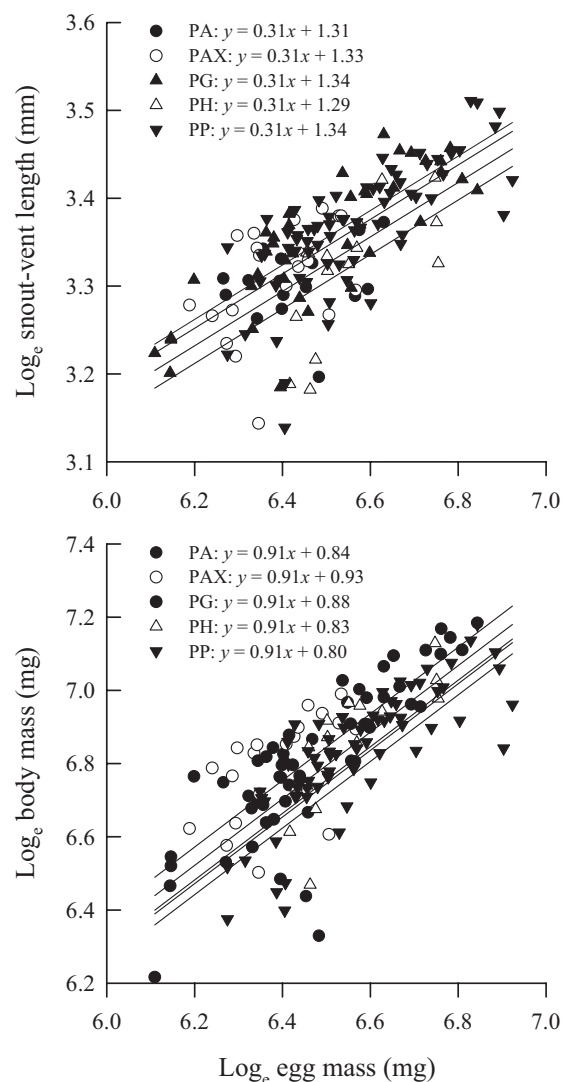


Figure 1 Linear regressions of hatchling snout-vent length and body mass against egg mass. All data were \log_e transformed. ●: *P. albolineatus* (PA); ○: *P. axillaries* (PAX); ▲: *P. grumgrzimailoi* (PG); △: *P. helioscopus* (PH); ▼: *P. przewalskii* (PP). Regression lines in each plot were adjusted for five species with a common slope to facilitate comparisons. The corrected regression equations are given in the figure.

Table 1 Embryonic stages at laying and incubation lengths at three temperatures in five oviparous *Phrynocephalus* lizards.

Species	Embryonic stages at laying	Incubation length (d)		
		24 °C	28 °C	32 °C
<i>P. albolineatus</i>	31 (2)	44.2 ± 1.8 (6)	33.6 ± 0.4 (5)	26.2 ± 0.2 (5)
		41–53	33–35	26–27
<i>P. axillaries</i>	28–30 (3)	49.2 ± 1.7 (6)	38.3 ± 0.9 (3)	28.3 ± 0.3 (10)
		45–57	37–40	27–30
<i>P. grumgrzimailoi</i>	30–31 (9)	55.8 ± 1.0 (16)	34.6 ± 1.1 (7)	26.9 ± 0.3 (15)
		43–61	32–40	25–29
<i>P. helioscopus</i>	29–30 (3)	49.0 ± 1.8 (6)	34.3 ± 0.5 (4)	26.2 ± 0.2 (5)
		43–53	33–35	26–27
<i>P. przewalskii</i>	30–32 (18)	48.4 ± 0.4 (20)	32.3 ± 0.5 (25)	25.7 ± 0.3 (24)
		43–51	29–37	23–28

Data on incubation length are expressed as mean ± SE and range, and numbers in parentheses are sample sizes.

although the mean incubation length at any given temperature may vary among species or even among populations that differ in phylogeny, egg size, embryonic stage at laying and/or distribution (Ji *et al.*, 2002; Du *et al.*, 2010b; Lin *et al.*, 2010; Li *et al.*, 2012, 2013;

Sun *et al.*, 2013). Incubation temperatures of 24 °C, 28 °C and 32 °C did not have any differential effects on hatchling morphology in the five species studied herein. This result is similar to that for other oviparous lizards inhabiting climatically challenging regions as

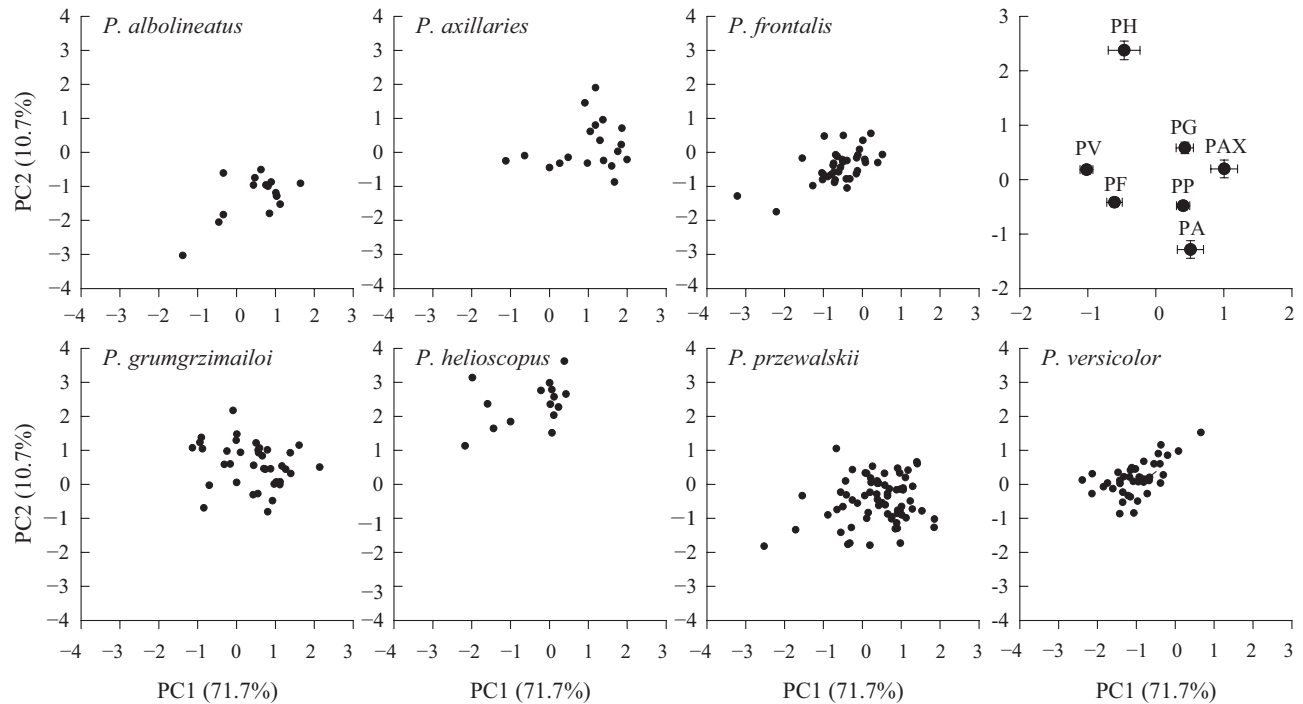


Figure 2 Positions of hatchlings of seven oviparous *Phrynocephalus* lizards in the space defined by the first two axes of a principal components analysis based on seven hatchling morphological variables. Size effects were removed using residuals from the regressions of corresponding variables on egg mass at laying (for hatchling SVL and mass) or hatchling SVL (for the other five variables). The plot in the upper right corner shows mean values (\pm) for factor scores on the first and second axes of the seven species. See Table 3 for definitions for PA, PAX, PF, PG, PH, PP and PV.

Table 2 Descriptive statistics, expressed as mean \pm SE and range, for hatchling size and morphology of five oviparous *Phrynocephalus* lizards.

N	<i>P. albolineatus</i>	<i>P. axillaries</i>	<i>P. grumgrzimailoi</i>	<i>P. helioscopus</i>	<i>P. przewalskii</i>	
	16	19	38	15	69	
Snout–vent length (mm)	27.2 \pm 0.3 24.5–29.2	27.4 \pm 0.4 23.2–29.6	28.6 \pm 0.4 24.2–32.2	27.7 \pm 0.5 24.1–30.7	29.1 \pm 0.2 23.1–33.5	$F_{4,151} = 3.83, P < 0.006$ PA ^{ab} , PAX ^{ab} , PG ^a , PH ^b , PP ^a
Body mass (mg)	858.9 \pm 36.7 561–1100	899.5 \pm 28.2 667–1086	953.1 \pm 33.2 501–1319	955.4 \pm 40.9 645–1248	935.1 \pm 18.4 587–1313	$F_{4,151} = 5.51, P < 0.001$ PA ^{ab} , PAX ^a , PG ^a , PH ^{ab} , PP ^b
Head length (mm)	8.4 \pm 0.1 7.5–9.0	8.3 \pm 0.1 7.0–9.1	8.4 \pm 0.1 7.0–9.3	8.3 \pm 0.1 7.0–9.2	8.8 \pm 0.1 7.4–9.9	$F_{4,151} = 7.92, P < 0.0001$ PA ^a , PAX ^{ab} , PG ^b , PH ^{ab} , PP ^a
Head width (mm)	6.4 \pm 0.1 5.8–7.0	6.7 \pm 0.1 5.8–7.2	6.8 \pm 0.1 5.7–7.6	7.1 \pm 0.1 6.1–7.9	6.9 \pm 0.05 5.9–7.9	$F_{4,151} = 15.94, P < 0.0001$ PA ^c , PAX ^b , PG ^{bc} , PH ^a , PP ^{bc}
Tail length (mm)	42.8 \pm 0.5 39–46	36.2 \pm 1.0 29–42	35.1 \pm 0.6 28–41	27.2 \pm 0.7 22–32	39.8 \pm 0.5 28–49	$F_{4,151} = 96.48, P < 0.0001$ PA ^a , PAX ^b , PG ^c , PH ^d , PP ^b
Fore–limb length (mm)	9.6 \pm 0.2 8.6–10.9	10.6 \pm 0.2 8.5–12.4	10.4 \pm 0.1 8.6–11.9	10.1 \pm 0.2 8.2–11.5	10.8 \pm 0.1 8.1–12.3	$F_{4,151} = 8.30, P < 0.0001$ PA ^c , PAX ^a , PG ^{bc} , PH ^{bc} , PP ^{ab}
Hind–limb length (mm)	16.8 \pm 0.2 15.9–18.0	17.3 \pm 0.3 14.6–20.1	17.0 \pm 0.2 14.1–19.0	15.1 \pm 0.3 12.2–16.7	17.6 \pm 0.2 14.3–20.6	$F_{4,151} = 24.03, P < 0.0001$ PA ^{ab} , PAX ^a , PG ^b , PH ^c , PP ^b

Statistical results of one-way ANCOVA with egg mass (for hatchling SVL and mass) or hatchling SVL (for the other five variables) are given in the table. PA: *P. albolineatus*; PAX: *P. axillaries*; PG: *P. grumgrzimailoi*; PH: *P. helioscopus*; PP: *P. przewalskii*. Regression lines in each plot were adjusted for five species with a common slope to facilitate comparisons. Species with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$; a > b > c).

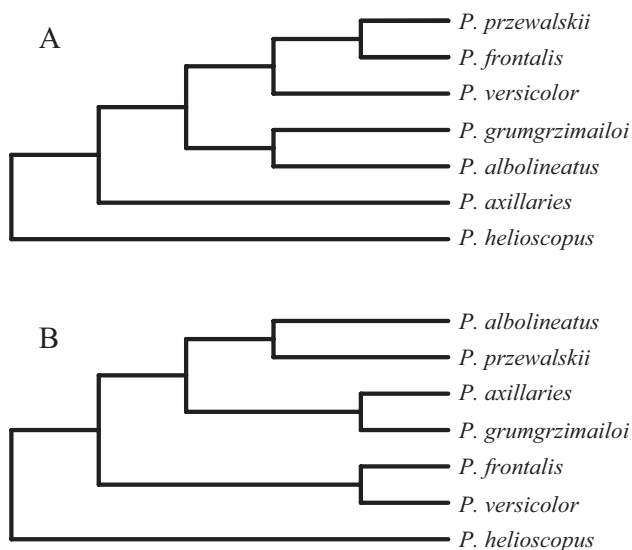


Figure 3 Trees represent the phylogenetic relationship of seven *Phrynocephalus* lizards. A: the tree proposed by Guo and Wang (2007) based on mitochondrial DNA data; B: the tree established in this study based on morphological data.

do *Phrynocephalus* lizards (Hao *et al.*, 2006; Li *et al.*, 2013), and adds evidence for the existence of a widened range of suitable incubation temperatures in *Phrynocephalus* lizards (Qu *et al.*, 2011b; Tang *et al.*, 2012).

It has been reported that incubation length does not differ between *P. frontalis* and *P. versicolor* (Qu *et al.*, 2011b), nor between *P. przewalskii* and *P. versicolor* (Tang *et al.*, 2012). However, data from this study and those reported previously for *P. przewalskii* (Table 1; Zeng *et al.*, 2013) show that incubation length may vary among and within species. In *P. przewalskii*, for example, incubation lengths are longer in populations where females lay eggs with less advanced (developed) embryos at earlier DH stages (Zeng *et al.*, 2013). Data collected in seven oviparous *Phrynocephalus* species found in China show that incubation length is negatively related to DH stage at laying, although such a relationship is not significant at 24 °C because only two or three eggs have been dissected to identify DH stage at laying in three of the seven species (Table 4). Among the four *Phrynocephalus* species (*P. frontalis*, *P. grumgrzimailoi*, *P. przewalskii* and *P. versicolor*) where no fewer than nine eggs have been dissected, the mean DH stage at laying is earliest in *P. grumgrzimailoi* (~30) and latest in *P. frontalis* and *P. versicolor* (~31) and, as expected, the mean incubation length is longest in *P. grumgrzimailoi* (~35 d at 28 °C) and shortest in the latter two species (~32 d at 28 °C) (Qu *et al.*, 2011b; Table 1). These results provide an inference that the changeover from DH stage 30 to 31 shortens the mean incubation length at 28 °C by

about 3 d. A similar trend has also been observed in *P. przewalskii* in which the changeover from DH stage 30 to 31 shortens the mean incubation length at 28 °C by about 3 d (Zeng *et al.*, 2013). Taken together, available data show that embryonic stage at laying is a causal factor of inter- and intra-specific variation in incubation length in *Phrynocephalus* lizards.

Consistent with an earlier study of other two oviparous *Phrynocephalus* species (*P. frontalis* and *P. versicolor*; Qu *et al.*, 2011b), data from this study show that the five species studied herein differ morphologically at hatching (Table 2). After accounting for egg mass, hatchling SVL was significantly longer in *P. grumgrzimailoi* and *P. przewalskii* than in *P. helioscopus*, and hatchling mass was significantly greater in *P. axillaries* and *P. grumgrzimailoi* than in *P. przewalskii* (Figure 1; Table 2). The latter finding is of particular interest because it suggests that, as has been reported for *Takydromus septentrionalis* (Du *et al.*, 2010a) and *P. frontalis* (Qu *et al.*, 2011b), females of *P. axillaries* and *P. grumgrzimailoi* are able to further increase the mass of their hatchlings by investing more dry materials and thus more energy into the egg without increasing the size of their eggs.

Data from this study and those reported previously for *P. frontalis* and *P. versicolor* show that oviparous *Phrynocephalus* lizards found in China differ from each other in hatchling morphology (Figure 2; Table 3). The phylogenetic relationship inferred from hatchling morphology is consistent with the relationship inferred

Table 3 Loading of the first two axes of a principal components analysis on seven hatchling morphological variables.

	Factor loading	
	PC 1	PC 2
Snout-vent length	0.893	0.111
Body mass	0.780	0.382
Head length	0.880	0.027
Head width	0.834	0.359
Tail length	0.721	0.614
Fore-limb length	0.906	0.013
Hind-limb length	0.896	0.296
Variance explained (%)	71.7	10.7
Factor scores on PC1: $F_{6,228} = 28.79$, $P < 0.0001$; PA ^a , PAX ^a , PF ^c , PG ^{ab} , PH ^c , PP ^b , PV ^c		
Factor scores on PC2: $F_{6,228} = 65.97$, $P < 0.0001$; PA ^d , PAX ^b , PF ^c , PG ^b , PH ^a , PP ^c , PV ^b		

Size effects are removed using residuals from the regressions on egg mass at laying (for hatchling SVL and mass) or hatchling SVL (for the other five variables). Variables with the main contribution are in bold face font. PA: *P. albolineatus*; PAX: *P. axillaries*; PF: *P. frontalis*; PG: *P. grumgrzimailoi*; PH: *P. helioscopus*; PP: *P. przewalskii*; PV: *P. versicolor*. Species with different superscripts differ significantly (Tukey's test, $\alpha = 0.05$; a > b > c > d).

Table 4 Linear regression models of incubation length (independent variable) against embryonic stage (dependent variable) at three constant temperatures, according to data from this study and Qu *et al.* (2011b) on seven species of oviparous *Phrynocephalus* lizards.

Incubation temperature (°C)	Regression models		Statistical results		
	Slope	Intercept	r^2	$F_{1,5}$ values	P levels
24	-2.27	117.20	0.22	1.40	0.290
28	-2.20	100.95	0.70	11.48	0.020
32	-1.56	73.28	0.66	9.70	0.026

from mitochondrial DNA data in two aspects: one is that the differences between *P. helioscopus* and the other six species are greater than those between any other species pair, and the other is that *P. frontalis* is more closely related to *P. versicolor* than to any other species (Figure 3). However, our morphological data do not support another two conclusions from the studies using mitochondrial DNA data: (1) there is no clear distinction between *P. albolineatus* and *P. grumgrzimailoi* (Guo and Wang, 2007); and (2) there is no species boundary between *P. frontalis* and *P. przewalskii* (Gozdzik and Fu, 2009). Morphologically, *P. albolineatus* is more similar to *P. przewalskii*, and *P. axillaries* is more related to *P. grumgrzimailoi* (Figure 3).

In summary, our data show that temperatures within the range of 24–32 °C affect incubation length but not hatchling morphology in all five *Phrynocephalus* lizards studied herein. By combining data from this study with those reported previously, we find that embryonic stage at laying is a causal factor of inter- and intra-specific variation in incubation length, and that the seven oviparous *Phrynocephalus* lizards found in China differ from each other in hatchling morphology. The phylogenetic relationship inferred from hatchling morphology is not always consistent with the currently known relationship inferred from mitochondrial DNA data. Data from this study and those reported previously allow the conclusion that any *Phrynocephalus* species may have its unique position along the axis defined by hatchling morphology.

Acknowledgements The work was carried out in compliance with the current laws on animal welfare and research in China, and was supported by grants from the National Natural Science Foundation of China (31071910 and 31200282), Priority Academic Program Development of Jiangsu Higher Education Institutions and High Academic Talent Foundation of Nanjing Forestry University (GXL201306). We thank Ce CHEN, Tianbao FU, Hong LI, Hongliang LU, Zhuyuan ZHANG

and Zongshi ZHOU for help in the field.

References

- Barabanov A. V., Ananjeva N. B. 2007. Catalogue of the available scientific species group names for lizards of the genus *Phrynocephalus* Kaup, 1825 (Reptilia, Sauria, Agamidae). *Zootaxa*, 1399: 1–57
- Du W. G., Ji X., Zhang Y. P., Lin Z. H., Xu X. F. 2010a. Geographic variation in offspring size of a widespread lizard (*Takydromus septentrionalis*): Importance of maternal investment. *Biol J Linn Soc*, 101: 59–67.
- Du W. G., Warner D. A., Langkilde T., Robbins T., Shine R. 2010b. The physiological basis of geographic variation in rates of embryonic development within a widespread lizard species. *Am Nat*, 176: 522–528
- Dufaure J. P., Hubert J. 1961. Table de développement du lézard vivipare: *Lacerta (Zootoca) vivipara* Jacquin. *Arch Anat Micr Morph Exp*, 50 : 309–328
- Gozdzik A., Fu J. Z. 2009. Are toad-headed lizards *Phrynocephalus przewalskii* and *P. frontalis* (Family Agamidae) the same species? Defining species boundaries with morphological and molecular data. *Russ J Herpetol*, 16: 107–118
- Guo X. G., Wang Y. Z. 2007. Partitioned Bayesian analyses, dispersal–vicariance analysis, and the biogeography of Chinese toad-headed lizards (Agamidae: *Phrynocephalus*): A re-evaluation. *Mol Phylogenet Evol*, 45: 643–662
- Hao Q. L., Liu H. X., Ji X. 2006. Phenotypic variation in hatchling Mongolian racerunners (*Eremias argus*) from eggs incubated at constant versus fluctuating temperatures. *Acta Zool Sin*, 52: 1049–1057
- Ji X., Braña F. 1999. The influence of thermal and hydric environments on incubating eggs and embryonic use of energy and nutrients in the wall lizard *Podarcis muralis*. *Comp Biochem Physiol A*, 124: 205–213
- Ji X., Huang H. Y., Hu X. Z., Du W. G. 2002. Geographic variation in female reproductive characteristics and egg incubation in the Chinese skink, *Eumeces chinensis*. *Chin J Appl Ecol*, 13: 680–684
- Ji X., Wang Y. Z., Wang Z. 2009. New species of *Phrynocephalus* (Squamata, Agamidae) from Qinghai, Northwest China. *Zootaxa*, 1988: 61–68
- Jin Y. T., Li L. Q., Liu N. F. 2013. Elevation-related variation in life history traits among *Phrynocephalus* lineages on the Tibetan Plateau: Do they follow typical squamate ecogeographic patterns? *J Zool*, 290: 293–301
- Li C., Lian X., Bi J. H., Fang H., Maul T. L., Jiang Z. G. 2011. Effects of sand grain size and morphological traits on running speed of toad-headed lizard *Phrynocephalus frontalis*. *J Arid Environ*, 75: 1038–1042
- Li H., Ding G. H., Zhou Z. S., Ji X. 2013. Fluctuations in incubation temperature affect incubation duration but not morphology, locomotion and growth of hatchlings in the sand lizard *Lacerta agilis* (Lacertidae). *Acta Zool (Stockholm)*, 94: 11–18
- Li H., Wang Z., Chen C., Ji X. 2012. Does the variance of incubation temperatures always constitute a selective force for

- the origin of reptilian viviparity? *Curr Zool*, 58: 812–819
- Lin L. H., Ma X. M., Li H., Ji X.** 2010. Phenotypic variation in hatchling Chinese ratsnakes (*Zaocys dhumnades*) from eggs incubated at constant temperatures. *J Therm Biol*, 35: 28–33
- Noble D., Qi Y., Fu J. Z.** 2010. Species delineation using Bayesian model-based assignment tests: A case study using Chinese toad-headed agamas (genus *Phrynocephalus*). *BMC Evol Biol*, 10: 197
- Qi Y., Wan H. F., Gu H. J., Wang Y. Z.** 2011. Do displays and badges function in establishing the social structure of male toad-headed lizards, *Phrynocephalus vlangalii*? *J Ethol*, 29: 381–387
- Qu Y. F., Gao J. F., Mao L. X., Ji X.** 2011a. Sexual dimorphism and female reproduction in two sympatric toad-headed lizards, *Phrynocephalus frontalis* and *P. versicolor* (Agamidae). *Anim Biol*, 61: 139–151
- Qu Y. F., Li H., Gao J. F., Ji X.** 2011b. Embryonic thermosensitivity and hatchling morphology differ between two coexisting lizards. *Acta Oecol*, 37: 375–380
- Qu Y. F., Li H., Gao J. F., Xu X. F., Ji X.** 2011c. Thermal preference, thermal tolerance and the thermal dependence of digestive performance in two coexisting *Phrynocephalus* lizards (Agamidae), with a review of species studied. *Curr Zool*, 57: 684–700
- Shu L., Zhang Q. L., Qu Y. F., Ji X.** 2011. Thermal tolerance, selected body temperature thermal dependence of food assimilation and locomotor performance in the Qinghai toad-headed lizard, *Phrynocephalus vlangalii*. *Acta Ecol Sin*, 30: 2036–2042
- Sun B. J., Li S. L., Xu X. F., Zhao W. G., Luo L. G., Ji X., Du W. G.** 2013. Different mechanisms lead to convergence of reproductive strategies in two lacertid lizards (*Takydromus wolteri* and *Eremias argus*). *Oecologia*, 172: 645–652
- Sun Y. Y., Du Y., Yang J., Fu T. B., Lin C. X., Ji X.** 2012. Is the evolution of viviparity accompanied by a relative increase in maternal abdomen size in lizards? *Evol Biol*, 39: 388–399
- Tang X. L., Yue F., Ma M., Wang N. B., He J. Z., Chen Q.** 2012. Effects of thermal and hydric conditions on egg incubation and hatchling phenotypes in two *Phrynocephalus* lizards. *Asian Herpetol Res*, 3: 184–191
- Wang Y. J., Li W. H., Zhao W., Yu N. N., Liu, N. F.** 2011. Geographic variation in clutch size and egg size for the lizard *Phrynocephalus przewalskii* (Squamata: Agamidae). *Asian Herpetol Res*, 2: 97–102
- Wang Y. Z., Fu J. Z.** 2004. Cladogenesis and vicariance patterns in the toad-headed lizard *Phrynocephalus versicolor* species complex. *Copeia*, 2004: 199–206
- Wang Y. Z., Zeng X. M., Fang Z. L., Liu Z. J., Wu G. F., Papenfuss T. J., Macey R. J.** 1996. A new species of *Phrynocephalus*: *Phrynocephalus zetanensis* sp. nov. *Zool Res*, 17(1): 27–29
- Wang Y. Z., Zeng X. M., Fang Z. L., Wu G. F.** 1999. Study on the relationships of classification, phylogenetics, and distribution of the genus *Phrynocephalus* spp. (Sauria: Agamidae), with the paleogeographic changes during Cenozoic era in Tibet plateau. *Zool Res*, 20 (3): 178–185
- Wang Y. Z., Zeng X. M., Fang Z. L., Wu G. F., Liu Z. J., Papenfuss T. J., Macey R. J.** 2002. A valid species of the genus *Phrynocephalus*: *P. putjatia* and a discussion on taxonomy of *Phrynocephalus hongyuanensis* (Sauria: Agamidae). *Acta Zootaxon Sin*, 27: 372–383
- Wang Z., Lu H. L., Ma L., Ji X.** 2013a. Viviparity in high-altitude *Phrynocephalus* lizards is adaptive because embryos cannot fully develop without maternal thermoregulation. *Oecologia*, doi:10.1007/s00442-013-2811-8
- Wang Z., Lu H. L., Ma L., Ji X.** 2013b. Differences in thermal preference and tolerance among three *Phrynocephalus* lizards (Agamidae) with different body sizes and habitat use. *Asian Herpetol Res*, 4: 214–220
- Zeng Z. G., Zhao J. M., Sun B. J.** 2013. Life history variation among geographically close populations of the toad-headed lizard (*Phrynocephalus przewalskii*): Exploring environmental and physiological associations. *Acta Oecol*, 51: 28–33
- Zhang X. D., Ji X., Luo L. G., Gao J. F., Zhang L.** 2005. Sexual dimorphism and female reproduction in the Qinghai toad-headed lizard *Phrynocephalus vlangalii*. *Acta Zool Sin*, 51: 1006–1012
- Zhao K. T.** 1999. *Phrynocephalus* Kaup, 1825. In Zhao E. M., Zhao K. T., Zhou K. Y. (Eds.), *Fauna Sinica, Reptilia (Squamata: Lacertilia)*, Vol. 2. Beijing: Science Press, 151–193